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IMPROVED FLUORESCENT ASSAY SENSITIVITY USING SILVER ISLAND FILMS: FLUORESCEIN ISOTHIOCYANATE-LABELED ALBUMIN AS AN EXAMPLE

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We have studied enhancement of the fluorescence of fluorescein isothiocyanate (FITC), bound to albumin and near an annealed silver island film, as a function of the distance between the protein molecules and the metal. As the intermediate spacer layer between the albumin and the silver substrate, we used multilayer films based on polyelectrolytes. The maximum nine- fold enhancement coefficient for the fluorescence of FITC corresponds to a thickness of the intermediate layer of ≈4 nm, or three layers of the polyelectrolyte. In this case, we observe a significant decrease in the average photoluminescence decay time for the label near the silver film compared with a dielectric medium.

Key words: *enhanced fluorescence; island silver film; intermediate layer (spacer); bovine serum albumin labeled by fluorescein isothiocyanate.*

Introduction. Modification of the photoluminescence (FL) of atoms and molecules located near nanostructured metallic surfaces [1–6] is an important and promising phenomenon in the optics of nanostructures. Detailed study of these processes is important both for understanding the fundamental aspects of the interaction between light and matter and for solving applied problems in nanotechnology and spectroscopy, in particular for designing new efficient luminophores and improving the sensitivity of the fluorescent method in enzyme immunoassays.

In contrast to giant Raman scattering (GRS), widely studied since the 1980s, where typically maximum signal enhancement occurs when the molecules are adsorbed directly on rough metallic substrates, enhancement of the luminescence signal occurs when the molecule is positioned at some distance from a metallic substrate, as was predicted theoretically in [7]. This effect is due to two specific processes: 1) enhancement of the luminescence of the molecules due to excitation of surface plasmons in the metal and redistribution of the density of photon states; 2) quenching of the luminescence signal as a consequence of nonradiative energy transfer from the molecule to the metal.

To date, surface-enhanced optical phenomena (giant Raman scattering, enhancement of luminescence, enhancement of second harmonic generation) and their mechanisms and the factors affecting the enhancement are under intense study, and the search is underway to find the conditions for obtaining the greatest possible enhancement factor. In particular, in order to study the fluorescence as a function of the distance between the molecule and the metal, different intermediate dielectric layers (spacers) are used (proteins, phospholipids, organic acids, amines, and their derivatives [8, 9], deposited on the metal substrate by the Langmuir–Blodgett method; polymers (polymethylmethacrylate, polylysine [8]), quartz [9], making it possible to controllably vary the distance from the luminophore to the metal.

It was suggested earlier that multilayer structures based on polyelectrolytes be used as the intermediate layer [4], where the process of deposition onto the metal surface is based on electrostatic self-assembly of oppositely charged polymers from appropriate aqueous solutions. In this work we have used polyelectrolyte multilayer structures

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to study the sensitivity of enhanced fluorescence to the distance between an FITC-labeled protein and an island silver film.

Experimental procedure. Island silver films are known to be effective substrates for giant Raman scattering [10]. The procedure for preparing them involves vacuum sputtering of silver films of thickness 14 nm on glass substrates of size 1 × 2 cm, followed by annealing at 340°C. In order to study the morphology of the films obtained, we used a Nanotechnology P4 AFM/STM atomic force microscope (AFM). The absorption spectra were obtained on a Cary 500 spectrophotometer (Varian); the fluorescence spectra were obtained on a DFS spectrometer (LOMO) with excitation by emission from an argon laser at a wavelength of λ = 488 nm. The time-correlated photon counting method was used to measure the fluorescence kinetics. A PLS-450 light-emitting diode (PicoQuant GmbH) was used as the pulse excitation source. The excitation pulse duration was ~750 psec, the repetition frequency was ~2.5 MHz, the excitation wavelength was 460 nm. The luminescence kinetics were modeled by the sum of decaying exponentials:

$$I(t) = \sum_i a_i \exp(-t/\tau_i) .$$

The results were processed to determine the parameters of the fluorescence kinetics a_i and τ_i using T900 software (Edinburgh Instruments). The average decay time was found from the formula

$$\langle\tau\rangle = \sum_i a_i\tau_i^2 \Big/ \sum_i a_i\tau_i ,$$

where τ_i is the decay time; the a_i are the corresponding coefficients in the two-exponential model for the decay. In this case, the contribution from each component was expressed as

$$\text{Rel} = a_i\tau_i \Big/ \sum_i a_i\tau_i .$$

Polydiallyldimethylammonium chloride (polycation, M = 200,000) and sodium polystyrene sulfonate (polyanion, M = 70000) were used as the polyelectrolytes for deposition of the dielectric layer. The method involved soaking each metallic substrate first in a solution of the polycation at a concentration of 1 g/L with addition of 0.5 M NaCl for 20 min, and then in a solution of the polyanion of the same concentration. The procedure was repeated several times until the required thickness of the multilayer film was achieved.

For the fluorescent bioassay, we used bovine serum albumin labeled with fluorescein isothiocyanate (BSA-FITC, Fluka) with mole ratio BSA:FITC = 1:10. Since the isoelectric point of albumin is 4.7 and in neutral aqueous solution the molecules have a negative charge, the albumin can electrostatically interact with the polycation. An aqueous solution of the protein of concentration $3.7\cdot10^{-8}$ M was mixed with the polydiallyldimethylammonium chloride, and from this solution the fluorophore was deposited onto the intermediate layer by soaking. Thus the last layer for each sample was the polycation with BSA-FITC additive.

Results and discussion. Fig. 1 shows a typical image of the silver film annealed at 340°C, obtained using the atomic force microscope. The surface relief is represented by hemispherical islands of average height ≈58 nm. The optical density spectra for this silver film and the FITC bound to the albumin overlap considerably (Fig. 2, spectra 1, 2); the excitation wavelength of 488 nm is close to the plasmon resonance in the metallic film, which ensures effective excitation of the fluorophore and enhancement of the fluorescence. The silver film was sputtered onto only half the surface of the glass substrates, which allowed us to compare the intensity of fluorescence for FITC bound to albumin and deposited on a silver film and on a dielectric glass surface for different thicknesses of the intermediate layer. The polyelectrolytes used as the intermediate layer do not absorb in this region of the spectrum, and cannot act as donors/acceptors of electronic excitation for the studied BSA-FITC complexes.

The fluorescent assay results are shown in Fig. 3. As we see, the fluorescence intensity of the label is sensitive to the distance between the silver island film and the protein, and we observe a maximum for three layers (curve 1), which corresponds to a thickness of ≈4 nm [11]. This result is different from the previously obtained value of ≈10

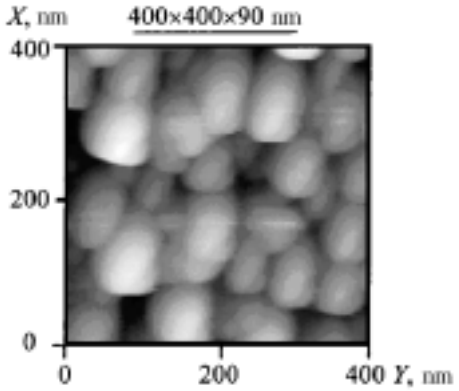

Fig. 1 AFM image of silver island film sputtered on a glass surface and annealed at 340°C.

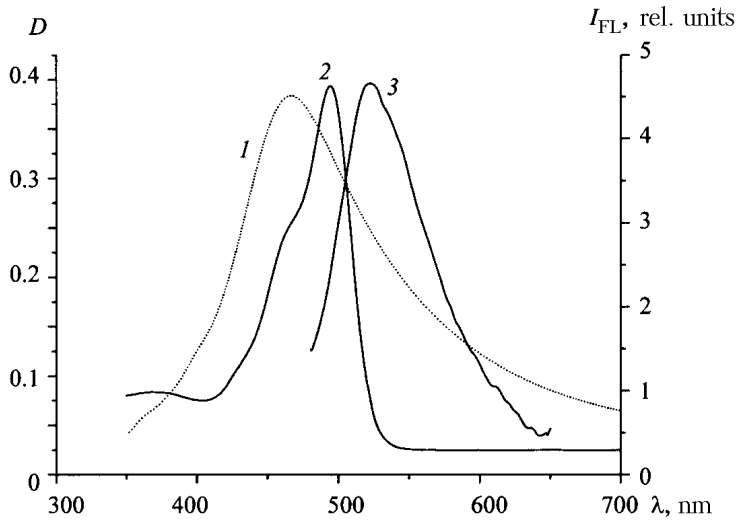

Fig. 2 Absorption spectra of annealed silver island film (1), an aqueous BSA-FITC solution (2), and the fluorescence spectrum of FITC bound to albumin and adsorbed on a silver film (3); λ_{ex} = 430 nm.

nm for CdSe/ZnS nanocrystals deposited on a colloidal gold film [4], which may be due to the different metal-fluorophore system, in particular the much greater size of the albumin molecules (4 × 14 nm) compared with the semiconductor nanocrystals (2–4 nm). Furthermore, a complication of this biological fluorescent complex is that on the average there are 10 FITC molecules per BSA molecule, and there is some distribution of the distances between probes on the protein surface within its size limits, hindering exact determination of the distance between the substrate and all the fluorescent probes. The results we obtained for the greatest enhancement for a thickness of the dielectric interlayer of 4 nm should be considered as the result for the average signal from many fluorescent probes, with a near-field distribution at distances from 4 nm to 18 nm.

Curve 2 in Fig. 3 shows only a slight difference (≤18%) of the fluorescence intensity of the label for BSA-FITC adsorbed on the glass for different thicknesses of the polyelectrolyte layer. This fact is very important for using polyelectrolytes as a separating layer, since it allows us to avoid artifacts in estimating the fluorescence enhancement factor.

For the sample with three layers of the polyelectrolytes, since it exhibited the maximum nine-fold enhancement of the fluorescence signal, we also measured the luminescence decay kinetics. As noted above, each protein

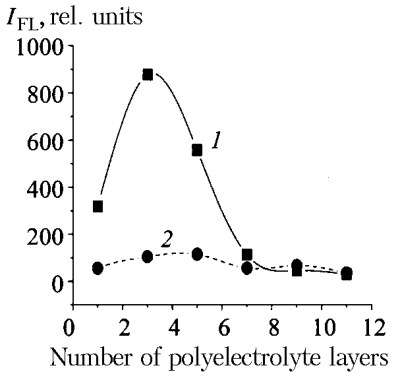

Fig. 3 Intensity of fluorescence of FITC bound to albumin vs. the number of layers of the polyelectrolyte film deposited on a silver island film (1) and on a glass substrate (2); λ_{ex} = 488 nm, λ_{det}= 525 nm.

TABLE 1 Biexponential Analysis of Fluorescence Kinetics for FITC Molecules Bound to Albumin, Adsorbed Through an Intermediate Spacer Layer (Three Polyelectrolyte Layers) on Glass and Silver Substrates

Substrate	τ_i, nsec	a_i	Rel, T	$\langle\tau\rangle$, nsec	χ^2
Glass	0.48 2.30	0.806 0.194	45.36 54.64	1.46	1.1
Silver	0.30 1.59	0.956 0.044	80.74 19.26	0.55	1.4

molecule contains 10 FITC molecules. Furthermore, association of the labeled proteins on the polymer occurs when they interact with the polycation. We see that for this reason, some concentration quenching and a decrease in the average fluorescence decay time occur for the FITC bound to albumin on the polyelectrolytes compared with an aqueous solution of FITC, which according to [12] is 3.8–4.2 nsec. Furthermore, the decay kinetics for the fluorescence intensity is best described by a biexponential law rather than by the monoexponential law which is typical for FITC in aqueous solutions. Table 1 gives the results of analysis of the fluorescence kinetics for the FITC molecules bound to the protein and deposited on a silver and a glass substrate. It is quite obvious that for BSA-FITC on a silver film, compared with BSA-FITC on a glass substrate there is typically a decrease in each of the components of the decay time and the average decay time and also an increase in the contribution of the fast component to the temporal behavior of the decay.

The fluorescence decay time (τ) and the quantum yield (Q) are determined [13] by the probabilities of radiative (Γ) and nonradiative (k) transitions:

$$\tau = \frac{1}{\Gamma + k}, \quad Q = \frac{\Gamma}{\Gamma + k}.$$

Therefore a nine-fold increase in the fluorescence intensity in the case of silver, also accompanied by a decrease in the decay time, may be associated with an increase in the probability of radiative transitions on the molecules in the presence of the metal, if we assume that k does not change in this case. However, the presence of the metal also may change the probability of nonradiative transitions, and so it is impossible to exactly determine the change in the fluorescence quantum yield of the molecules in the presence of the metal from the available data. On the other hand, enhancement of fluorescence may be connected with more efficient excitation of the molecules due to excitation of surface plasmons, and may be induced by strong local electromagnetic fields on the surface of the metallic film [2, 6]. A more detailed study of the mechanisms for enhancement of fluorescence will be the subject of our further investigations.

Conclusion. We have experimentally established that annealed silver island films with multilayer polyelectrolyte layers deposited on them as an intermediate dielectric spacer can be used as substrates for improving the efficiency of fluorescent assay of weakly fluorescent biological entities (proteins, DNA, etc.). The maximum enhancement factor is obtained when the labeled entity is placed at a distance of ≈4 nm from the metal, which corresponds to three layers of the polyelectrolyte film. The dielectric layers used and the method for depositing the fluorophore on them may also be used to deposit any water-soluble charged molecules at a controllable distance from a metallic substrate in studying surface-enhanced optical phenomena (giant Raman scattering, luminescence, second harmonic generation).

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